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Immunisation of Common Marmosets With Vaccinia Virus Expressing Epstein-Barr Virus (EBV) gp340 and Challenge With EBV

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Epstein-Barr virus (EBV) is the cause of infectious mononucleosis and is associated with a variety of life-threatening diseases in humans. Therefore the development of an effective vaccine is an important objective. Many of the initial studies of vaccine efficacy analyse the ability of vaccine preparations to prevent the induction of lymphomas in cottontop tamarins by the B95-8 strain of EBV. We used a vaccinia virus recombinant expressing gp340, vMA1, tested previously in the cotton-top tamarin, to evaluate a common marmoset model in which the challenge virus, M81, resembles more closely the wild-type strains of EBV in the general population than does the standard B95-8 strain. We characterised the M81 strain of EBV with respect to the sequence of its gp340/220 gene and in regard to the presence of a region deleted in B95-8. Replication of the challenge virus in the group vaccinated with vMA1 was decreased when compared to control groups.

KEY WORDS: vaccinia recombinant, M81, gp340, membrane antigen

INTRODUCTION

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with a number of diseases of lymphoid and epithelial origin. These diseases include Burkitt's lymphoma [de Thé et al., 1978], lymphoproliferative conditions arising in immunosuppressed transplant patients and in individuals infected with the human immunodeficiency virus [Hanto et al., 1981; Thomas and Crawford, 1989], oral hairy leukoplakia [Greenspan et al., 1989; Greenspan et al., 1985], and nasopharyngeal carcinoma [zur Hausen et al., 1970]. Increasingly EBV is being implicated in other malignancies such as Hodgkins lymphoma and gastric cancer [Herbst et al., 1990; Imai et al., 1994; Labrecque et al., 1995].

This range of life-threatening human diseases has been the motivation for development of an EBV vaccine that might modify the effects of infection. The cotton-top tamarin has proved to be an invaluable model for the design and evaluation of experimental Epstein-Barr virus vaccine candidates [Cleary et al., 1985; Epstein et al., 1985, 1986; Finerty et al., 1994, 1992; Morgan et al., 1988a,b, 1989]. Recombinant viruses, sub-unit vaccines, and features of novel vaccine preparations and adjuvants have all been evaluated in this model. However, only a few breeding colonies are available, thereby limiting the number of experiments that can be undertaken, and the outcome of challenge, a lymphoma, is more appropriate to the study of EBV-driven lymphomas than to natural infection by EBV.

Infection of the common marmoset with the M81 strain of EBV [de Thé et al., 1970; Desgranges et al., 1976] is followed by responses similar to those commonly seen in man [Wedderburn et al., 1984], in particular lymphocytosis, heterophile antibody, and long-term production of antibody to virus antigens [de Thé et al., 1980; Wedderburn et al., 1984]. Similar results can be achieved with larger doses of the B95-8 strain of EBV [Emini et al., 1986; Felton et al., 1984]. The long-term EB virus–host interactions are established essentially without clinical disease, a situation which is similar to natural infection in the majority of cases. Permissive natural infection was suggested by the seroconversion of virus capsid antigen (VCA)-negative animals that were housed with seropositive individuals [Cox et al., 1996]. These parallels between experimental and natural infection persuaded us to characterise the model further and evaluate it with

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TABLE I. Protocol for Experimental Vaccination of Common Marmosets With vMA1

Animal	Vaccinations (weeks −10 and −5)	EBV challenges (weeks 0 and 12)	CySA 1 (weeks 36–40)	CySA 2 (weeks 83–87)
519 ♀ 533 ♂ 540 ♂ 532 ♀	1. PBS 2. PBS	5×10^4 ID_{50} (M81 virus)	40 mg/kd/pd in Complan (32-day course)	60 mg/kd/pd in Complan (32-day course)
526 ♂ 532 ♂ 534 ♀	1. vTK-16 (5×107 pfu) 2. vTK-16 (2×108 pfu)	5×10^4 ID_{50} (M81 virus)	40 mg/kd/pd in Complan (32-day course)	60 mg/kd/pd in Complan (32-day course)
530 ♂ 538 ♀ 539 ♀ 541 ♀	1. vMA1 (5×107 pfu) 2. VMA1 (2×108 pfu)	5×10^4 ID_{50} (M81 virus)	40 mg/kd/pd in Complan (32-day course)	60 mg/kd/pd in Complan (32-day course)

a recombinant vaccinia virus vaccine preparation that *had already* been used successfully in the cottontop tamarin model. A similar vaccinia recombinant is also under evaluation in human volunteers [Gu et al., 1995].

MATERIALS AND METHODS

Animals

Callithrix jacchus. The common marmosets used in this study were part of a closed colony to which there had been no additions since 1970.

Vaccinia Viruses

vMA1 [Mackett and Arrand, 1985; Morgan et al., 1988b] is a recombinant vaccinia virus expressing the EBV gp340 gene product from the 7.5K early/late vaccinia virus promoter. The gene for gp340 was inserted into the thymidine kinase locus of the virus which consequently was functionally TK negative. Therefore we used a TK-negative virus, vTK-16, derived from the same parent strain as vMA1 [Weir et al., 1982; Weir and Moss, 1983] as a control.

Epstein-Barr Virus

EBV was obtained from the common marmoset cell line M81 [Desgranges et al., 1976]. Virus was concentrated from culture supernatants by centrifugation [Johnston et al., 1990].

Vaccination and Challenge

Eleven common marmosets (6 males and 5 females), aged between 33 and 58 weeks, were vaccinated as described in Table I. Intradermal (i.d.) vaccination was considered to be preferable to scarification in an experimental situation, as each animal should receive the same amount of vaccine, a result that can not be guaranteed by the sacrification method. Four animals were given two injections of vMA1 (i.d.) 10 and 5 weeks before EBV challenge. Four other animals were given similar injections (i.d.) with vTK-16. These latter animals were used as controls to establish the effects, if any, which a non-recombinant vaccinia might have.

Three common marmosets developed small vesicles following the first vaccination with 5×10^7 plaque-forming units (p.f.u.) in 50 μl (the largest being in 539♀, measuring 5 × 7 mm). The other five animals developed areas of pallor, surrounding a small area of erythema, peaking 9–12 days post-vaccination. Due to this mild response, each animal was given a second, higher dose of vaccinia (2×10^8 pfu in 100 μl). This resulted in a typical secondary response, with erythema, 2 days after vaccination. The final four control animals were given PBS. Animals were infected with EBV "orally" by injections into the palatine tonsils, the back of the tongue, and sublingually, under ketamine anaesthesia (10 mg/kg i.m.; Vetalar, Park Davis). All animals received two oral challenges of EBV, on weeks 0 and 12.

Immunosuppression

Thirty-six and 82 weeks after the initial EBV challenge, each animal received a 32-day course of Cyclosporin A (CySA), in order to determine the possible effect of immunosuppression on the levels of EBV infection. The first course (CySA 1) used material dissolved in olive oil mixed with Complan and fed to the animals daily at 40 mg/kg. The second course (CySA 2) used commercial CySA (Sandimmun, Sandoz), again mixed with Complan and administered to the animals in the same way, at a dose of 60 mg/kg/diem.

Blood and Saliva Samples

Blood for haematological investigations was taken under ketamine anaesthesia. Saliva was obtained following pilocarpine stimulation (1 mg/kg pilocarpine nitrate (Sigma) in saline, intradermally), under anaesthesia with alfadolone alfaxalone (1–1.5 mg/kg Saffan, Glaxovet). "Whole mouth fluid" (WMF) was obtained by inserting 0.3 ml of distilled water into the mouth, under ketamine anaesthesia, and removing the fluids.

Serology

Following immunisation, antibodies to VCA and early antigens were estimated by direct immunofluorescence [Bauer, 1983; Henle and Henle, 1966] and recorded for a 2½-year period. Antibodies to vaccinia virus were assayed in triplicate by enzyme-linked immunosorbent assay (ELISA) as described previously [Morgan et al., 1988b]. Antibodies to gp 340 were assayed over the first 54 weeks of the experiment by ELISA essentially as described [Randle and Epstein, 1984] using gp340/220

TABLE II. Summary of Conserved Amino Acid Sequences and Differences Between Strains of EBV*

```
B958   MEAALLVCQY TIQSLIHLTG EDPGFFNVEI PEFPFYPTCN VCTADVNVTI NFDVGGKKHQ LDLDFGQLTP
Ag876                  Q  R            L      A          A             K  N    L
M81

B958   HTKAVYQPRG AFGGSENATN LFLLELLGAG ELALTMRSKK LPINVTTGEE QQVSLESVDV YFQDVFGTMW
Ag876                                                    I
M81

B958   CHHAEMQNPV YLIPETVPYI KWDNCNSTNI TAVVRAQGLD VTLPLSLPTS AQDSNFSVKT EMLGNEIDIE
Ag876
M81                                                                          Q

B958   CIMEDGEISQ VLPGDNKFNI TCSGYESHVP SGGILTSTSP VATPIPGTGY AYSLRLTPRP VSRFLGNNSI
Ag876
M81

B958   LYVFYSGNGP KASGGDYCIQ SNIVFSDEIP ASQDMPTNTT DITYVGDNAT YSVPMVTSED ANSPNVTVTA
Ag876
M81

B958   FWAWPNNTET DFKCKWTLTS GTPSGCENIS GAFASNRTFD ITVSGLGTAP KTLIITRTAT NATTTTHKVI
Ag876
M81

B958   FSKAPESTTT SPTLNTTGFA DPNTTTGLPS STHVPTNLTA PASTGPTVST ADVTSPTPAG TTSGASPVTP
Ag876                        A
M81

                ] [
B958   SPSPWDNGTE SKAPDMTSST SPVTTPTPNA TSPTPAVTTP TPNATSPTPA VTTPTPNATS PTLGKTSPTS
Ag876                  ----  ----------      S
M81        R           ----  ----------                G       N

B958   AVTTPTPNAT SPTLGKTSPT SAVTTPTPNA TSPTLGKTSP TSAVTTPTPN ATGPTVGETS PQANATNHTL
Ag876            -  ------      P           I                                    T
M81              -  ----------  ----------  -------      P            S

                                                                          ] [
B958   GGTSPTPVVT SQPKNATSAV TTGQHNITSS STSSMSLRPS NSPETLSPST SDNSTSHMPL LTSAHPTGGE
Ag876      S        P                                  I
M81                 P       D         R               SI    -----  ----

B958   NITQVTPASI STHHVSTSSP APRPGTTSQA SGPGNSSTST KPGEVNVTKG TPQPNATSPQ APSGQKTAVP
Ag876            T
M81

B958   TVTSTGGKAN STTGGKHTTG HGARTSTEPT TDYGGDSTTP RPRYNATTYL PPSTSSKLRP RWTFTSPPVT
Ag876                                                T
M81                                        D

B958   TAQATVPVPP TSQPRFSNLS MLVLQWASLA VLTLLLLLVM ADCAFRRNLS TSHTYTTPPY DDAETYV*
Ag876
M81
```

*The inferred amino acid sequence of gp340/220 from B95-8(type A) is shown in single letter code on the top line and compared to gp340/220 from AG876 (type B) and M81. Only differences between the inferred sequences are shown. - indicates amino acids missing relative to B95-8, and the sequence between][indicates amino acids absent from gp220 due to splicing.

derived from a bovine papillomavirus expression vector system and adsorbing 0.1 μg of gp340/220 per well [Conway et al., 1989]. Results are expressed as the mean O.D. of a 1:10 dilution of serum from which background O.D. had been subtracted.

Serum from vaccinated animals was not tested for the presence of virus-neutralising activity, as previous experiments using the tamarin model showed that the detection of virus-neutralising activity did not necessarily correlate with protection [Epstein et al., 1986], and also that animals that did not show significant levels of virus-neutralising activity may nevertheless have been protected [Morgan et al., 1988b].

PCR and Sequence Analysis

Primers used for detection of M81 virus DNA, LLW1, and LLW2 [Labrecque et al., 1995] were based on the B95-8 sequence [Baer et al., 1984] of the *Bam*HI W fragment bp 505 and 740: LLW1 5′-CCATGTAAGCTTGCCTCGAC-3′. LLW2 5′-GCCTTAGATCTGGCTCTTTG-3′. Details of the PCR protocol and removal of inhibitory activity in WMF with Chelex 100 are given elsewhere [Cox et al., 1996]. A/B typing of M81 DNA was performed as described previously [Chen et al., 1992]. Primer sets detailed in Table I, based on B95-8 sequence, were used to amplify the M81 gp340/220 gene. Amplified DNA was sequenced by the Sanger chain termination method using fluorescent dye tagged dideoxynucleotides [Pepper et al., 1996] and an Applied Biosystems 373A automated DNA sequence machine.

RESULTS

The best-characterized vaccine antigens for an anti-EBV vaccine are the major membrane glycoproteins gp340 and gp220. These glycoproteins are the products of a single open reading frame with an in-frame splice

TABLE III. Anti-Vaccinia and Anti-gp340 Antibody Titres in Vaccinated Marmosets Before Challenge With EBV*

Animal	Anti-vaccinia (week −6)	Anti-vaccinia (Week −1)	Anti-gp340 (Week −6)	Anti-gp340 (Week −1)
(PBS)				
519 ♂	—	—	—	—
533 ♀	—	—	—	—
540 ♀	—	—	—	—
543 ♂	—	—	—	—
(vTK-16)				
526 ♂	1:25	1:250	—	—
532 ♂	1:250	1:2500	—	—
534 ♀	1:250	1:2500	—	—
(vMA1)				
530 ♂	1:25	1:250	—	1:25
538 ♀	1:25	1:250	1:25	1:25
539 ♀	1:250	—	1:10	1:10
541 ♂	1:25	1:250	—	1:25

*Antibody levels were determined as indicated in Materials and Methods. The dilution that gave an O.D. reading of 0.15 above the control is indicated.

[Beisel et al., 1985; Biggin et al., 1984]. Monoclonal antibodies and polyclonal antisera to gp340 neutralise EBV, and it has been shown that purified gp340 as well as gp340 expressed in an adenovirus vector [Epstein et al., 1985; Ragot et al., 1993] can protect cotton-top tamarins from lymphomas induced by the B95-8 strain of EBV. The recombinant vaccinia virus vMA1 which expresses the gp340 gene from the B95-8 strain of EBV induces anti-gp340 antibody in vaccinated rabbits and will protect cotton-top tamarins from EBV-induced lymphomas [Morgan et al., 1988b]. However, because the gp340 gene was derived from the B95-8 strain of EBV whle the challenge virus in the common marmoset model was the M81 strain, we felt it was important to characterise the M81 genome, and in particular the M81 gp340 gene.

Analysis of M81 DNA

Differences in biological activity between B95-8 and M81 have been reported [Desgranges et al., 1979], and consequently we decided to analyse several important features of the M81 genome. This included determining the EBV type to which M81 belongs, characterising a region of DNA present in wild-type viruses but deleted in B95-8 and establishing the sequence of the M81 gp340/220 gene. The missing region in B95-8 codes for three proteins in the Raji "strain" of EBV [Parker et al., 1990]. Southern blot analysis using probes for the region present in Raji but absent in B95-8 showed that M81, like Raji, contains extra DNA with the potential to encode three proteins (data not shown). A slight difference was noted between Raji and M81 in that the *Bam*HI restriction site between the I_b and I_c fragments was absent.

The polymerase chain reaction (PCR) analysis of the EBNA2 region of M81 DNA using type-specific primers [Chen et al., 1992] showed that the M81 strain, like B95-8, is EBV type A (type 1), the most common type detectable in the population. A sequence polymorphism where an A is substituted for by a T at position 49613 [Baer et al., 1984] in the B95-8 sequence was noted.

We went on to sequence the gp340/220 gene of M81 and compared this to the B95-8 (type A) and AG876 (type B) sequences [Biggin et al., 1984; Lees et al., 1993]. The DNA sequence is deposited with the EMBO data base ref ×9910E Table II shows a summary of the conserved amino acids and the differences between these strains of EBV. The sequence of the splice donor and acceptor sites is conserved, along with the high proline/serine/threonine content of the repeat region that is spliced out to give gp220. The repeat region of M81 is different from B95-8 and AG876 and more like that of other strains of virus present in the general population [Lees et al., 1993]. A single repeat is missing, and there is also a deletion relative to other strains between amino acids 635 and 643. This would predict that the molecular weight of gp220 is very similar in all strains but that gp340 of M81 is 51 amino acids smaller than that of B95-8. These data could explain at the molecular level differences reported between gp340 from different isolates [North et al., 1980]. Thirteen base pair changes between B95-8 and M81 outside the repeat region resulted in four amino acid differences with a cluster of differences in the region spliced out in gp220. The 5′ end signal sequence is maintained, as is the CD21-binding domain. It is thought that the major, conformationally sensitive, virus-neutralising epitope resides within the 5′ 181 amino acids of the molecule [Tanner et al., 1988] and the single amino acid change relative to B95-8 in the 5′ 450 amino acids of M81 is unlikely to affect this epitope particularly as the change is a conservative one. This inference is supported by the observation that the virus-neutralising monoclonal antibody 72A1 still recognises M81 cells (data not shown).

Vaccination Studies

Eleven common marmosets (6 males and 5 females), aged between 33 and 58 weeks, were vaccinated as described in Table I. Four weeks after the first and second vaccinations, serum from each animal was analysed for the presence of anti-vaccinia and gp340 antibodies, as determined by ELISA (see Table III; Fig. 1). Both the

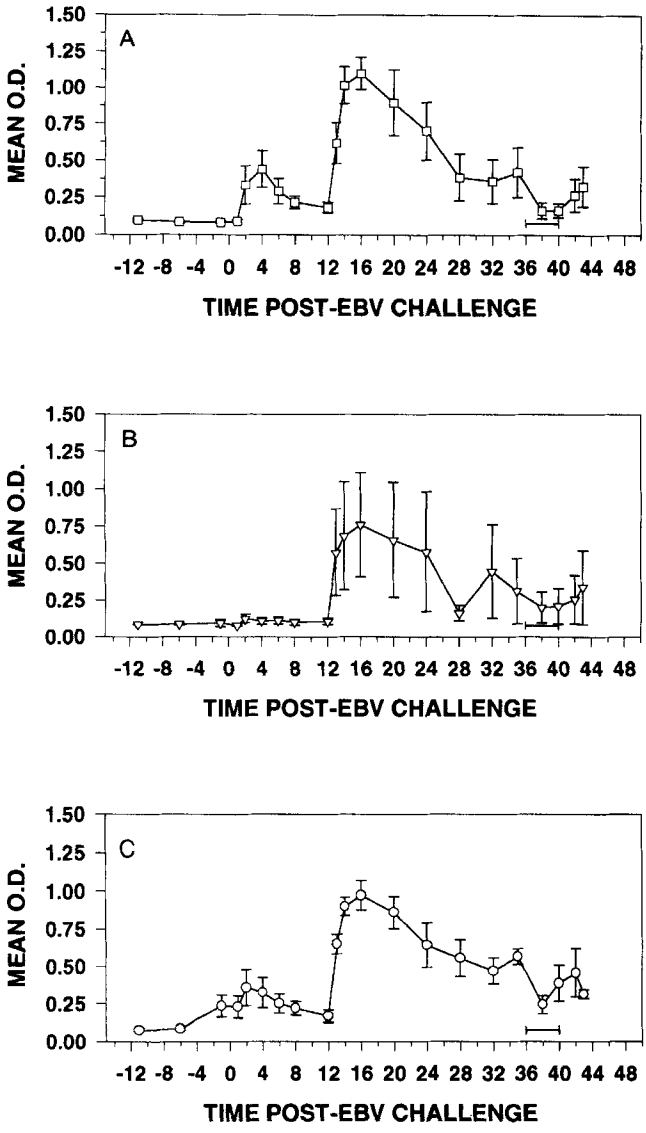

Fig. 1. The mean O.D. (± standard error) of an anti-gp340–specific ELISA test of sera collected from marmosets "vaccinated" with PBS (**A**; squares), vTK⁻16 (**B**; triangles), or vMA1 (**C**; circles) are shown. Marmosets received an oral EBV challenge on weeks 0 and 12 and a 32-day course of cyclosporin A between weeks 36 and 40 (⊢⊣). Immunisation was at weeks −10 and −5.

vTK⁻16- and vMA1-vaccinated animals had detectable levels of anti-vaccinia antibodies after 4 weeks, and in most cases higher levels were recorded 4 weeks after the second vaccination. Two of four animals given vMA1 developed low titres of anti-gp340 after the first vaccination, and four of four animals did so 4 weeks after the second vaccination.

Anti-gp340 Antibody Levels

The mean O.D. (± standard error) of an ELISA test for anti-gp340 antibodies from sera collected from the PBS or vTK⁻16 controls and from vMA1-vaccinated animals was analysed over a longer period. The results are shown in Figure 1A, B, and C, respectively.

Following EBV challenge, significant levels of anti-gp340 antibody were detected in the group receiving PBS (Fig. 1A). These levels increased strongly, following the second challenge with EBV (at week 12). In contrast, animals receiving vTK⁻16 (Fig. 1B) did not develop significant levels of anti-gp340 antibodies until after the second EBV challenge (the large range in standard error is a consequence of marmoset 534♀ remaining anti-gp340 negative). CySA treatment caused anti-gp340 antibody levels to rise in both groups. Marmosets vaccinated with vMA1 (Fig. 1C) had detectable levels of anti-gp340 antibodies present in their sera following vaccination. However, post-EBV challenge, these levels did not differ significantly from the two control groups.

Anti-VCA Immunofluorescence

The mean (± standard error) anti-VCA antibody titre of PBS and vTK⁻16 controls, and vMA1-vaccinated animals are shown in Figure 2A, B, and C, respectively.

Three of four marmosets vaccinated with vMA1, expressing gp340, developed positive antibody titres using P3HR1 cells during vaccination (see Fig. 2C). As the membranes of the P3HR1 cells, which were used to detect VCA, express gp340, it is not surprising that these cells should stain following incubation with serum from vaccinated animals. The exception was marmoset 530♂, which remained VCA negative until after EBV challenge. Following EBV challenge, the levels of anti-VCA antibodies detected in sera from vMA1-vaccinated animals were very similar to that seen in the PBS- and vTK⁻16-treated animals (Fig. 2A,B); titres increased following the first and second EBV challenge, and persisted thereafter. It was noted that about 10 weeks after the completion of the first course of CySA, VCA titres increased, while in general, the second course had no demonstrable effect.

Anti-EA Immunofluorescence

The anti-EA antibody titres of marmosets treated with PBS or vaccinated with vTK⁻16 and vMA1 are shown in Figure 3A, B, and C, respectively.

Analysis of sera from PBS-treated animals revealed that one animal had a persistent EA titre (543♂), two were positive sporadically for EA (533♀ and 519♂), while 540♀ remained negative throughout (Fig. 3A). Similar analysis of vTK⁻16-vaccinated animals revealed that 532♂ and 526♂ developed persistent EA titres, while 534♀ was positive sporadically (Fig. 3B). In contrast, none of the vMA1-vaccinated animals developed a persistent EA titre, although 539♀ was positive on several occasions, and 538♀ was also briefly positive (Fig. 3C). Similar results were obtained using an ELISA based on the EBV thymidine kinase (part of the EA complex) (data not shown).

Following EBV infection of the common marmoset not all infected animals develop anti-EA antibody titres. Some, although highly VCA positive, never do so, whereas many others only develop anti-EA months or years after infection [Cox et al., 1996; Wedderburn et al., 1984]. It was therefore interesting to note that 120

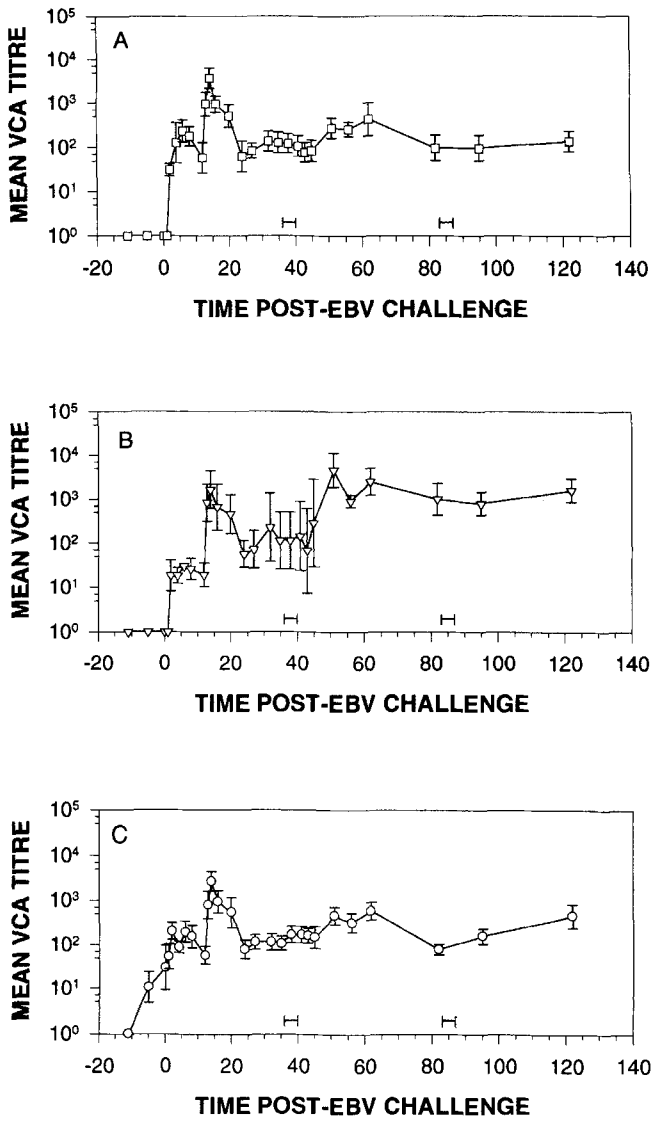

Fig. 2. The mean anti-VCA antibody titre (± standard error) of sera collected from marmosets vaccinated with PBS (**A;** squares), vTK⁻16 (**B;** triangles) or vMA1 (**C;** circles) are shown). Marmosets received an oral EBV challenge on weeks 0 and 12 and two 32-day courses of cyclosporin A between weeks 36 and 40 and weeks 83 and 87 (⊢⊣). Immunisation was at weeks −10 and −5.

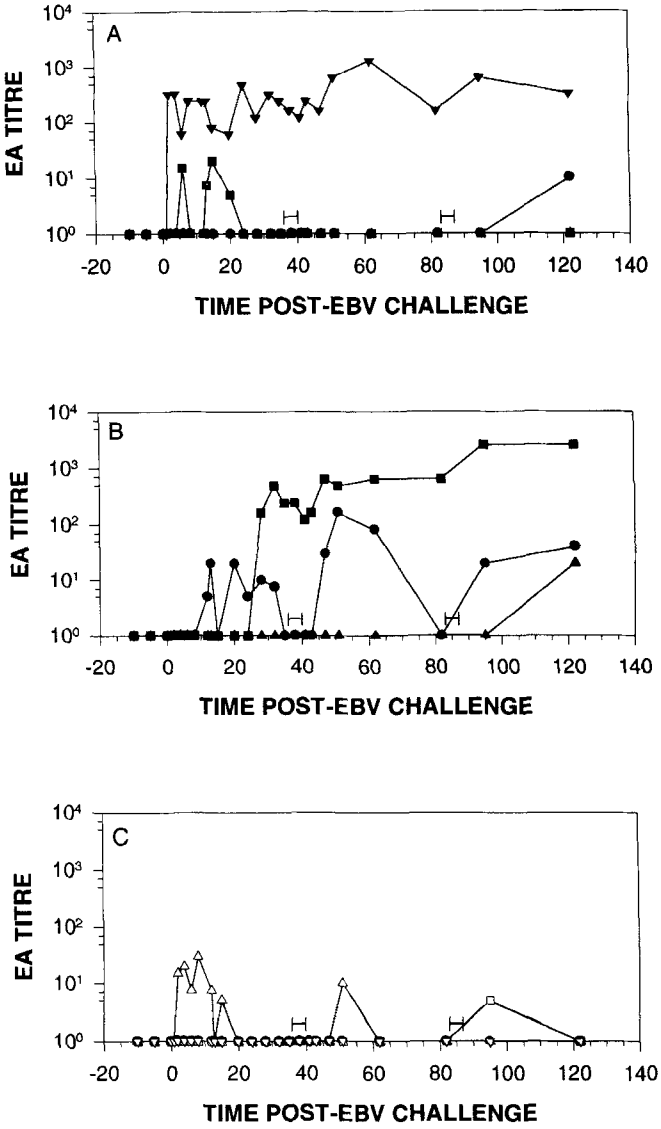

Fig. 3. **A:** The anti-EA antibody titres of sera collected from marmosets vaccinated with PBS are shown (circles, marmoset 519♂; triangles, marmoset 533 ♀; inverted triangles, marmoset 540♀; squares, marmoset 543♂). Marmosets received an oral EBV challenge on weeks 0 and 12 and two 32-day courses of cyclosporin A between weeks 36 and 40 and weeks 83 and 87 (⊢⊣). Immunisation was at weeks −10 and −5. **B:** The anti-EA antibody titres of sera collected from marmosets vaccinated with vTK⁻16 are shown (circles, marmoset 526♂; squares, marmoset 532 ♂; triangles, marmoset 534 ♀). Marmosets received an oral EBV challenge on weeks 0 and 12 and two 32-day courses of cyclosporin A between weeks 36 and 40 and weeks 83 and 87 (⊢⊣). Immunisation was at weeks −10 and −5. **C:** The anti-EA antibody titres of sera collected from marmosets vaccinated with vMA1 are shown (○ marmoset 530♂; □ marmoset 538♀; △ marmoset 539♀; ▽ marmoset 541♂). Marmosets received an oral EBV challenge on weeks 0 and 12, and two 32 day courses of cyclosporin A, between weeks 36 and 40 and weeks 83 and 87 (⊢⊣). Immunisation was at weeks −10 and −5.

weeks after EBV challenge, five of seven control animals were anti-EA postive, while all vMA1 vaccinated animals were negative.

Analysis of Buccal Fluids

The presence of EBV DNA in pilocarpine-induced saliva and in WMF was determined by slot-blot and PCR analysis (Table IV). Only oral samples collected at 4 and 8 weeks after the initial EBV infection and just prior to the sacrifice of the animals were available for analysis.

All marmosets, with the exception of 519♂, had high levels of EBV DNA detected in their saliva, as determined by slot-blot analysis with a radio-labelled *Bam*HI W-specific DNA probe, 4 weeks after the initial EBV challenge. Slot-blot analysis of saliva samples collected 4 weeks later (8 weeks post-challenge) were negative for all animals, when tested by the same procedure (data not shown). The high levels of EBV DNA detected in saliva following EBV infection could be due to viral shedding, or alternatively, material remaining from the rela-

TABLE IV. Analysis of Whole Mouth Fluid (WMF)*

Animal	WMF sample (Week 105)	WMF sample (Week 114)	WMF sample (Week 122)	Total
(PBS)				
519 ♂	+ve	+ve	—	2/3
533 ♀	—	+ve	+ve	2/3
540 ♀	+ve	—	+ve	2/3
543 ♂	—	+ve	+ve	2/3
+ ves	2/4	3/4	3/4	8/12
(vTK-16)				
526 ♂	—	+ve	+ve	2/3
532 ♂	+ve	—	—	1/3
534 ♀	+ve	+ve	+ve	3/3
+ves	2/3	2/3	2/3	6/9
(vMA1)				
530 ♂	+ve	—	—	1/3
538 ♀	—	+ve	—	1/3
539 ♀	+ve	—	+ve	2/3
541 ♂	—	—	+ve	1/3
+ves	2/4	1/4	2/4	5/12

*Marmosets were either treated with PBS or vaccinated with vTK-16 or vMA1, and WMF samples were analysed as described in Materials and Methods. Total figures correspond to the number of times a given animal had EBV DNA present in its WMF.

tively large amount of EBV DNA used in the oral infection. PCR analysis of WMF samples collected from paired marmosets in a mating experiment [Cox et al., 1996] did not indicate that EBV infection was followed by immediate shedding of a large amount of virus, although the amount of virus received in natural infection would probably be less than in experimental oral infection.

WMF samples collected on three occasions (weeks 105, 114, and 122) from marmosets inoculated with PBS were tested for the presence of EBV DNA. All samples were checked for the presence of inhibitors, and any inhibition found was removed by the use of Chelex® 100 [Cox et al., 1996]. Overall, eight of the 12 samples analysed from this group were found to contain EBV DNA. All animals were positive on two out of three occasions tested. Of the nine samples tested from TK⁻16-vaccinated animals, six were found to be positive for EBV DNA. Interestingly, WMF from 532♂ only tested positive on one occasion, even though this animal had a high persistent EA titre (Fig. 3B). In contrast, all samples tested from 534♀ were positive. This finding may be correlated with the sudden late development of an EA titre at this time in animal 534♀ (Fig. 3B). Only five of the 12 samples tested from the vMA1 group were found to contain EBV DNA. WMF from 530♂, 538♀, and 541♂ only tested positive on one occasion, while WMF from 539♀, the only animal from this group to develop an EA titre detectable on several occasions (Fig. 3C), was positive in two of three samples tested.

Overall, EBV DNA was detected, by PCR analysis, in 42% (5/12) of the WMF samples collected from animals vaccinated with vMA1, compared to 67% (14/21) of the WMF samples collected from the control groups (PBS and vTK⁻16).

DISCUSSION

We used a vaccinia virus recombinant expressing gp340, vMA1, tested previously in the cotton-top tamarin, to evaluate the common marmoset model for testing experimental anti-EBV vaccines. In this model the challenge virus, M81, is more closely related to wild-type strain of EBV circulating in the general population than the widely used B95-8 strain. The route of infection and dose of virus used is also closer to the natural situation than that used in the cotton-top tamarins. Experimental infection parallels natural challenge without overt clinical disease but with seroconversion and persistent infection. A number of experimental vaccines based on gp340 presented either as purified recombinant protein or as recombinant viruses have been described [Morgan et al., 1988a,b; Ragot et al., 1993]. All of these vaccines, including our recombinant vaccinia, use the gp340 gene isolated from the B95-8 strain of virus; as we were using the M81 strain, we felt it was important to characterise the gp340 gene of M81 to determine the extent of similarity between the two genes. Table I shows that only four amino acids differ between B95-8 and M81 gp340 outside of the repeat region. The differences are unlikely to affect the CD21 binding site, the splicing to form gp220, or the major virus neutralising epitope. It is, however, not possible to be sure that all T-cell epitopes are maintained, as very few have been defined [Ulaeto et al., 1988; Wallace et al., 1991]. However, the extremely close relationship between the two molecules is evident and provides a firm basis to expect cross protection in vaccination experiments between M81 and B95-8–derived gp340 recombinant products.

Historically vaccines have not prevented initial virus replication; they have, however, prevented clinical disease. It is in the light of this that attempts to vaccinate against agents that become latent or establish persistence should be viewed. In experimental lentivirus infection of primates successful vaccination has been reported in terms of delayed disease [Sutjipto et al., 1990], decreases in virus burden [Israel et al., 1994], or decrease

in virus isolation and not in prevention of infection. Here we report the successful vaccination of common marmosets against EBV in equivalent terms, decreases in early antigen being a surrogate marker for virus replication and DNA positivity in saliva being an indication of virus burden. In the context of primary infection with EBV in adolescence or at older ages a decrease in virus burden or replication may well be all that is required to prevent infectious mononucleosis (IM). Fifty percent of primary infections in adolescence resolve without obvious disease, and even a small decrease in virus load could allow the host's immune system to control infection more quickly and thereby prevent the clinical symptoms associated with the IM. As with traditional vaccines vaccination does not prevent infection, and in both the cotton-top tamarin and the common marmoset persistence of EBV occurs [Niedobitek et al., 1994; Wedderburn et al., 1984].

Infection of the common marmoset with the M81 strain of EBV offers a model for evaluating experimental EBV vaccines in which the clinical disease and the challenge virus parallels natural infection. Our results show that vaccination with a vaccinia recombinant expressing the EBV gp340 gene can decrease virus load compared to controls after oral challenge with EBV. The model will allow comparison of various strategies for vaccination against EBV including the use of purified gp340 [Cox et al., in preparation].

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REFERENCES

Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Seguin C, Tuffnell PS, Barrell BG (1984): DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310:207–211.

Bauer G (1983): Quantitative analysis of the cooperative effect between inducers of Epstein-Barr virus antigen synthesis. Journal of General Virology 64:1337–1346.

Beisel C, Tanner J, Matsuo T, Thorley-Lawson D, Kezdy F, Kieff E (1985): Two major outer envelope glycoproteins of Epstein-Barr virus are encoded by the same gene. Journal of Virology 54:665–674.

Biggin M, Farrell PJ, Barrell BG (1984): Transcription and DNA sequence of the BamHI L fragment of B95-8 Epstein-Barr virus. EMBO Journal 3:1083–1090.

Chen XY, Pepper S de V, Arrand JR (1992): Prevalence of the A and B types of Epstein-Barr virus DNA in nasopharyngeal carcinoma biopsies from southern China. Journal of General Virology 73: 463–466.

Cleary ML, Epstein MA, Finerty S, Dorfman RF, Bornkamm GW, Kirkwood JK, Morgan AJ, Sklar J (1985): Individual tumors of multifocal EB virus-induced malignant lymphomas in tamarins arise from different B-cell clones. Science 228:722–724.

Conway M, Morgan AJ, Mackett M (1989): Expression of Epstein-Barr virus membrane antigen gp340/220 in mouse fibroblasts using a bovine papillomavirus vector. Journal of General Virology 70: 729–734.

Cox C, Chang S, Karran L, Griffin B, Wedderburn N (1996): Persistent Epstein-Barr virus infection in the common marmoset (*Callithrax jacchus*) Journal of General Virology 77:1173–1180.

de Thé G, Ho HC, Kwan HC, Desgranges C, Favre MC (1970): Nasopharyngeal carcinoma (NPC). I. Types of cultures derived from tumour biopsies and non-tumorous tissues of Chinese patients with special reference to lymphoblastoid transformation. Inernational Journal of Cancer 6:189–206.

de Thé G, Geser A, Day NE, Tukei PM, Williams EH, Beri DP, Smith PG, Dean AG, Bronkamm GW, Feorino P, Henle W (1978): Epidemiological evidence for causal relationships between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature 274:756–761.

de Thé G, Dubouch P, Fontaine C, Wedderburn N, Carter RL, Edwards MB, Cohen B (1980): Natural antibodies to EBV-VCA antigens in common marmosets (Callithrix jacchus) and response after EBV inoculation. Intervirology 14:284–291.

Desgranges C, Lenoir G, de Thé G, Seigneurin JM, Hilgers J, Dubouch P (1976): In vitro transforming activity of EBV. I. Establishment and properties of two EBV strains (M81 and M72) produced by immortalized Callithrix jacchus lymphocytes. Biomedicine 25: 349–352.

Desgranges C, Lavoue MF, Patet J, de Thé G (1979): In vitro transforming activity of Epstein-Barr virus (EBV). II. Differences between M81 and B95-8 EBV strains. Biomedicine 30:102–108.

Emini EA, Luka J, Armstrong ME, Banker FS, Provost PJ, Pearson GR (1986): Establishment and characterization of a chronic infectious mononucleosis like syndrome in common marmosets. Journal of Medical Virology 18:369–379.

Epstein MA, Morgan AJ, Finerty S, Randle BJ, Kirkwood JK (1985): Protection of cottontop tamarins against Epstein-Barr virus-induced malignant lymphoma by a prototype subunit vaccine. Nature 318:287–289.

Epstein MA, Randle BJ, Finerty S, Kirkwood JK (1986): Not all potently neutralizing, vaccine-induced antibodies to Epstein-Barr virus ensure protection of susceptible experimental animals. Clinical and Experimental Immunology 63:485–490.

Felton SC, Hoffmann CC, Kreier JP, Glaser R (1984): Hematologic and immunologic responses in common marmosets (Calllithrix jacchus) infected with Plasmodium knowlesi and Epstein-Barr virus. Laboratory Animal Sciences 34:164–168.

Finerty S, Tarlton J, Mackett M, Conway M, Arrand JR, Watkins PE, Morgan AJ (1992): Protective immunization against Epstein-Barr virus-induced disease in cottontop tamarins using the virus envelope glycoprotein gp340 produced from a bovine papillomavirus expression vector. Journal of General Virology 73:449–453.

Finerty S, Mackett M, Arrand JR, Watkins PE, Tarlton J, Morgan AJ (1994): Immunization of cottontop tamarins and rabbits with a candidate vaccine against the Epstein-Barr virus based on the major viral envelope glycoprotein gp340 and alum. Vaccine 12:1180–1184.

Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Conant MA, Petersen V, Freese UK (1985): Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. New England Journal of Medicine 313:1564–1571.

Greenspan D, Greenspan JS, de Souza Y, Levy JA, Ungar AM (1989): Oral hairy leukoplakia in an HIV-negative renal transplant recipient. Journal of Oral Pathology and Medicine 18:32–34.

Gu SY, Huang TM, Ruan L, Miao YH, Lu H, Chu CM, Motz M, Wolf H (1995): First EBV vaccine trial in humans using recombinant vaccinia virus expressing the major membrane antigen. Developments in Biological Standardisation 84:171–177.

Hanto DW, Frizzera G, Purtilo DT, Sakamoto K, Sullivan JL, Saemundsen AK, Klein G, Simmons RL, Najarian JS (1981): Clinical spectrum of lymphoproliferative disorders in renal transplant recipients and evidence for the role of Epstein-Barr virus. Cancer Research 41:4253–4261.

Henle G, Henle W (1966): Immunofluorescence in cells derived from Burkitt's lymphoma. Journal of Bacteriology 91:1248–1256.

Herbst H, Niedobitek G, Kneba M, Hummel M, Finn T, Anagnostopoulos I, Bergholz M, Krieger G, Stein H (1990): High incidence of Epstein-Barr virus genomes in Hodgkin's disease. American Journal of Pathology 137:13–18.

Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, Tanaka S, Sato E, Osato T (1994): Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. Proceedings of the National Academy of Sciences USA 91:9131–9135.

Israel ZR, Edmonson PF, Maul DH, O'Neil SP, Mossman SP, Thiriart C, Fabry L, van Opstal O, Bruck C, Bex F, Burny A, Fultz PN, Mullins JI, Hoover EA (1994): Incomplete protection, but suppression of virus burden, elicited by subunit simian immunodeficiency virus vaccines. Journal of Virology 68:1843–1853.

Johnston DA, Knight AM, Naylor BA, Wedderburn N, Mitchell GH

(1990): Monoclonal antibodies from Epstein-Barr virus-transformed lymphocytes of common marmosets (Callithrix jacchus) immune to malaria. Journal of Immunological Methods 127:187–195.

Labrecque LG, Barnes DM, Fentiman IS, Griffin BE (1995): Epstein-Barr virus in epithelial cell tumors: a breast cancer study. Cancer Research 55:39–45.

Lees JF, Arrand JE, Peppers de V, Stewart JP, Mackett M, Arrand JR (1993): The Epstein-Bar virus candidate vaccine antigen gp340/220 is highly conserved between virus types A and B. Virology 195: 578–586.

Mackett M, and Arrand JR (1985): Recombinant vaccinia virus induces neutralising antibodies in rabbits against Epstein-Barr virus membrane antigen gp340. EMBO Journal 4:3229–3234.

Morgan AJ, Finerty JS, Lovgren K, Scullion FT, Morein B (1988a): Prevention of Epstein-Barr (EB) virus-induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into immune-stimulating complexes. Journal of General Virology 69:2093–2096.

Morgan AJ, Mackett M, Finerty S, Arrand JR, Scullion FT, Epstein MA (1988b): Recombinant vaccinia virus expressing Epstein-Barr virus glycoprotein gp340 protects cottontop tamarins against EB virus-induced malignant lymphomas. Journal of Medical Virology 25:189–195.

Morgan AJ, Allison AC, Finerty S, Scullion FT, Byars NE, Epstein MA (1989): Validation of a first-generation Epstein-Barr virus vaccine preparation suitable for human use. Journal of Medical Virology 29:74–78.

Niedobitek G, Agathanggelou A, Finerty S, Tierney R, Watkins P, Jones EL, Morgan AJ, Young LS, Rooney N (1994): Latent Epstein-Barr virus infection in cottontop tamarins. A possible model for Epstein-Barr virus infection in humans. American Journal of Pathology 145:969–978.

North JR, Morgan AJ, Epstein MA (1980): Observations on the EB virus envelope and virus-determined membrane antigen (MA) polypeptides. International Journal of Cancer 26:231–240.

Parker BD, Bankier A, Satchwell S, Barrell B, Farrell PJ (1990): Sequence and transcription of Raji Epstein-Barr virus DNA spanning the B95-8 deletion region. Virology 179:339–346.

Pepper S de V, Stewart JP, Arrand JR, Mackett M (1996): Murine gammaherpesvirus-68 encodes homologues of thymidine kinase and glycoprotein H: sequence, expression and characterization of pyrimidine kinase activity. Virology 219:475–479.

Ragot T, Finerty S, Watkins PE, Perricaudet M, Morgan AJ (1993): Replication-defective recombinant adenovirus expressing the Epstein-Barr virus (EBV) envelope glycoprotein gp340/220 induces protective immunity against EBV-induced lymphomas in the cottontop tamarin. Journal of General Virology 74:501–507.

Randle BJ, Epstein MA (1984): A highly sensitive enzyme-linked immunosorbent assay to quantitate antibodies to Epstein-Barr virus membrane antigen gp340. Journal of Virological Methods 9:201–208.

Sutjipto S, Pedersen NC, Miller CJ, Gardner MB, Hanson CV, Gettie A, Jennings M, Higgins J, Marx PA (1990): Inactivated simian immunodeficiency virus vaccine failed to protect rhesus macaques from intravenous or genital mucosal infection but delayed disease in intravenously exposed animals. Journal of Virology 64:2290–2297.

Tanner J, Whang Y, Sample J, Sears A, Kieff E (1988): Soluble gp350/220 and deletion mutant glycoproteins block Epstein-Barr virus adsorption to lymphocytes. Journal of Virology 62:4452–4464.

Thomas JA, Crawford DH (1989): Epstein-Barr virus associated B-cell lymphomas in AIDS and after organ transplantation. Lancet 1:1075–1076.

Ulaeto D, Wallace L, Morgan AJ, Morein B, Rickinson AB (1988): In vitro T cell responses to a candidate Epstein-Barr virus vaccine: human CD4+ T cell clones specific for the major envelope glycoprotein gp340. European Journal of Immunology 18:1689–1697.

Wallace LE, Wright J, Ulaeto DO, Morgan AJ, Rickinson AB (1991): Identification of two T-cell epitopes on the candidate Epstein-Barr virus vaccine glycoprotein gp340 recognized by CD4+ T-cell clones. Journal of Virology 65:3821–3828.

Wedderburn N, Edwards JM, Desgranges C, Fontaine C, Cohen B, de Thé G (1984): Infectious mononucleosis-like response in common marmosets infected with Epstein-Barr virus. Journal of Infectious Diseases 150:878–882.

Weir JP, Moss B (1983): Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. Journal of Virology 46:530–537.

Weir JP, Bajszar G, Moss B (1982): Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA. Proceedings of the National Academy of Sciences USA 79:1210–1214.

zur Hausen H, Holthausen H, Schulte H, Klein G, Henle W, Henle G, Clifford P, Santesson L (1970): EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature 228:1056–1058.